

## MODULATION OF LINE-1 REVERSE TRANSCRIPTASE

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This application is a continuation-in-part of prior Application No. 10/758,329, filed January 15, 2004, which claims the benefit of U.S. Provisional Application No. 60/440,988 filed January 15, 2003, and the text of applications 10/758,329 and 60/440,988 is incorporated by reference in its entirety herewith.

## FIELD OF THE INVENTION

The present invention is directed to the field of cancer therapy. Specifically, target molecules have been identified modulation of which regulates elongation of telomeres in telomerase negative cancerous cells. More particularly, it relates to the use of various inhibitor compounds that interfere with human L1 (Line-1) retrotransposon encoded reverse transcriptase (L1RT) for treating or preventing L1RT induced cancers. The invention also relates to screening methods for identifying pharmacologically active compounds that may be useful for treating L1RT-mediated proliferative diseases.

## BACKGROUND OF THE INVENTION

An asymmetry in the synthesis of leading and lagging DNA strands creates the "end problem" for replication of linear genomes<sup>8</sup>. To overcome this, eukaryotic chromosomes have specialized end structures, telomeres, consisting of TTAGGG repeats<sup>9</sup>. Telomerase is a ribonucleoprotein enzyme that elongates telomeres and therefore maintains chromosomal stability in majority of cancer cells during cell doubling. The gradual loss of DNA from the ends of telomeres during cell doubling has been implicated in the control of cellular proliferative potential in somatic cells<sup>10</sup>.

Normal cultured human cells have a limited replication potential in culture. Normal cells in culture replicate until they reach a discrete point at which population growth ceases. This is termed M1 stage and is caused by the shortening of a few telomeres to a size that leads to a growth arrest called cellular senescence. This stage can be bypassed in vitro by abrogation of the function of p53 and pRB human tumor suppressor genes. The cells then can proliferate until the telomeres have become critically shortened, which produces the M2 or crisis stage. The growth arrest in the M2 stage is caused by balance between the cell proliferation and cell death rate. At this stage, when most of telomeres are extremely short, end-to-end fusions and

chromosomal breakage-fusion cause marked chromosomal abnormalities and apoptosis. Under rare circumstances, a cell can escape M2 and become immortal by stabilizing the length of its telomeres. This occurs through the activation of the enzyme telomerase or an alternative mechanism of telomere lengthening (ALT).

5 Human germline<sup>2</sup> and the majority of cancer cells<sup>3</sup> express telomerase. Telomerase is a ribonucleoprotein enzyme that elongates telomeres and, therefore, maintains chromosomal stability in majority of cancer cells during cell doubling. Indeed, elongation of shortened telomeres by telomerase is a major mechanism of telomere maintenance in the human cancer cells. Inhibition of telomerase limits the  
10 growth of human telomerase positive cancer cells<sup>11</sup> by decreasing telomere length, these compounds diminish the ability of these cancer cells to proliferate. Reverse transcriptase inhibitors have been used previously to treat cancer. In in vitro tests, tumor cells treated with the reverse transcriptase inhibitors underwent apoptosis after 14 days.

15 Elongation of shortened telomeres by telomerase is a well known mechanism of telomere maintenance in the human cancer cells. However up to 30% of human tumors of different types do not express telomerase. The presence of ALT was reported in up to 30% of human tumors of different types, tumor-derived cell lines and human cell lines immortalized *in vitro*<sup>4,5,12,13</sup>, and up to 50% in some subsets of  
20 tumors and immortalized cell lines<sup>14</sup>.

Currently, strategies aimed at selectively treating the cancers from telomerase positive cells involve modulation of TERT function or length of telomeres by antisense strategy, dominant negative mutants or pharmacological agents (see, Bisoffi et al., Eur J Cancer, 1998, 34:1242-1249; Roth et al., Leukemia, 2003, 17:2410-2417;  
25 Damm et al., EMBO J., 2001, 20:6958-6968; U.S. Patents 6,294,332, 6,194,206, 6,156,763 and 6,046,307). Selective modulation (i.e., selective inhibition or promotion) of telomerase negative cancer cells may also be made possible if the target molecule(s) responsible for the lengthening of telomeres in such cells are known. Thus, there is need for identifying target molecules responsible for the lengthening of  
30 telomeres in telomerase negative cells and identifying agents for selectively interfering with the identified target molecules so that human tumors of types that do not express telomerase may also be prevented or treated.

## SUMMARY OF THE INVENTION.

It has now been found that a product of L1 (LINE-1) retrotransposon reverse transcriptase nucleic acid is associated with the lengthening and therefore  
5 maintenance of telomeres in certain cancer cells. Specifically, it has been found that interference with the expression of reverse transcriptase encoded by the L1 retrotransposon suppresses the elongation of telomeres in the cancer cells. More specifically, it has been found that interference with the expression of the L1 reverse transcriptase in telomerase negative cells leads to phenotypic manifestations such as  
10 telomere shortening, cell cycle arrest and apoptosis or cell death. It is believed that the reverse transcriptase is involved in maintaining telomeres probably by "slippage" mechanism of telomeric DNA synthesis and/or telomere end targeted L1 transposon retrotransposition.

Still more specifically, it has been found that treatment of the telomerase  
15 negative cells (ALT cells) with reverse transcriptase inhibitor 3'-azido-2',3'-dideoxythymidine (AZT) or suppression of L1 reverse transcriptase (L1RT) using antisense strategy induces progressive telomere loss, G2 phase arrest, chromosomal abnormalities and eventual cell death.

Accordingly, in one embodiment of the invention, a method is provided for  
20 treating tumors characterized by expression of L1RT and/or absence of telomerase expression. Interference with L1RT expression or activity will either directly result in cell death or will potentiate the effects of chemotherapeutic agents that ultimately kill cells through apoptosis. In particular, the invention provides a method for inhibiting proliferation of L1RT expressing cells having potential for continuous increase in cell  
25 number by administering inhibitors and antagonists of L1RT. For example, L1RT expression can be suppressed or down regulated by obtaining a DNA molecule having a cDNA sequence operably linked to a promoter such that it will be expressed in antisense orientation, the cDNA having all or part of the sequence of L1RT, and transfecting, with the DNA molecule, the L1RT cells with potential for uncontrolled  
30 proliferation. The inhibitor or antagonist is optionally administered with a pharmaceutically acceptable carrier.

In another embodiment of the invention, a method for prevention of a cancer in a person (e.g. a human) in need thereof is provided. The cancer is due to the presence in the human of cells showing alternative lengthening of telomeres induced or mediated by L-1 (LINE-1) retrotransposon encoded reverse transcriptase in the cells of the person. Lengthening of telomeres in cells induces a potential for continuous proliferation of such cells in the human body. The preventive method involves administration of a therapeutically effective amount of a composition to the person. The composition has an inhibitor or antagonist of the reverse transcriptase. The inhibitor or antagonist blocks the lengthening of telomeres in telomerase-negative cells, thereby inhibiting proliferation of L1RT expressing cells. Preferably, the inhibitor is one or more nucleoside analogs, or a pharmaceutically acceptable salt of such analogs. A liquid or solid food material is enriched with inhibitor or antagonist. The food product can be, for example, a functional food in the form of butter, margarine, biscuits, bread, cake, candy, confectionery, yogurt or another fermented milk product, or cereal suitable for consumption by humans. Alternatively, it can be a nutritional supplement, a nutrient, a pharmaceutical, food, a nutraceutical, a health food and/or a designer food. Periodically, the human is tested for the presence of ALT cells. The use of inhibitor or antagonist may be stopped once the ALT cells are no longer detected.

In another embodiment of the invention, a method is provided for screening candidate drugs or compounds to select drugs with potential for decreasing the rate of accumulation of tumor cells by incubating or treating cells expressing L1RT with a candidate drug and monitoring one or more desired biological effects the candidate drug(s) may have on the cells. If the candidate drug causes a desired biological effect, then the drug is selected. Particularly preferred biological effects in such a screening include progressive telomere loss, G2 phase arrest, chromosomal abnormalities or cancer cell death. The biological effects may also include inhibition of proliferation of telomerase negative cells transformed with various oncogenes such as, for example, *ras*.

The invention further provides methods and kits for detecting pathologically proliferating cells expressing L1RT. These and other embodiments of the invention will be described in more detail below.

## BRIEF DESCRIPTION OF THE DRAWINGS

**Figure 1** is a dot blot of total cellular RNA from ALT and telomerase positive cell lines with telomere specific probe. 1, U-2 OS. 2, Saos-2. 3, no RNA. 4, HEC-1. 5, HeLa.

5       **Figure 2** illustrates flow cytometry data showing decrease in telomere length, massive apoptosis and changes in cell cycle after 14 days of treatment of ALT cell lines with AZT. Telomere specific fluorescence in G2 phase of cell cycle in (a) Saos-2; (b) U-2 OS cells. Cell cycle distribution<sup>22</sup> in (c) Saos-2; (d) U-2 OS cells. Untreated cells – grey, treated – dark.

10       **Figure 3** illustrates flow cytometry data showing changes in DNA synthesis rate, cell cycle distribution and telomere length in U-2 OS cells treated with AZT for different amounts of time. a, b, c, d no treatment and treatment for 10, 17, and 40 days respectively. Cell cycle distribution<sup>24</sup> – left. Staining for BdU incorporation (FITC) and PI<sup>24</sup> – middle. Staining with PNA-FITC and PI – right. The numbers  
15 indicate telomere specific fluorescence measured in arbitrary units<sup>22</sup> in G1 and G2 phases respectively.

**Figure 4** illustrates PNA-FITC and PI staining flow cytometry data showing decrease in telomere length, massive apoptosis and changes in cell cycle after 14 days of ganciclovir treatment. (a) untreated U-2 OS cells; (b) ganciclovir treated U-2 OS  
20 cells.

**Figure 5** shows a schematic representation of L1 reverse transcriptase antisense targeting strategy.

**Figure 6** illustrates flow cytometry data showing changes in cell cycle distribution and telomere length in U-2 OS cells transfected for 40 days with L1  
25 targeted antisense construct. (a) no treatment; (b) sense construct; (c) antisense construct.

## DETAILED DESCRIPTION OF THE INVENTION

      The present invention discloses that LINE-1 (L1) retrotransposon encoded  
30 reverse transcriptase (L1RT) enzyme is involved in lengthening of telomeres in certain human cancer cells. Specifically, the present invention discloses that L1RT is involved in lengthening of telomeres in certain tumor tissues including telomerase

negative tumors and the tumor-derived cell lines, and identifies L1RT enzyme or the sequences encoding it as a target for controlling the proliferative properties of the tumor cells or inducing apoptosis of these cells.

The telomerase negative tumors and the tumor-derived cell lines are those that  
5 do not express or have the endogenous telomerase and yet show lengthening of telomeres, also referred to herein as alternative lengthening of telomeres (ALT). The L1RT mediated telomere lengthening in cells can be characterized by the presence of long and heterogeneous telomeres relative to the telomere lengthening mediated by telomerase. One skilled in the art would know how to determine the presence of long  
10 and heterogeneous telomeres characteristic of ALT in cells by carrying out, for example, TRF assay (see, Bryan et al., 1997, *Nature Medicine*, 3:1271-1274).

L1 reverse transcriptase, which is encoded by ORF2 of L1 retrotransposon, has already been characterized and its nucleic acid and protein sequences are known in the art (GeneBank GI: 5070620; Ostertag et al., 2000, Determination of L1  
15 retrotransposition kinetics in cultured cells, *Nucleic Acids Res.* 28, 1418-1423; Kimberland et al., 1999, Full-length human L1 insertions retain the capacity for high frequency retrotransposition in cultured cells, *Hum. Mol. Genet.* 8 (8), 1557-1560). In the present invention, it has been discovered that L1RT adds telomeric DNA repeats to chromosomes in telomerase negative cells.

20 Accordingly, in an aspect of the present invention, methods for preventing or treating disorders caused by the presence of inappropriately or pathologically proliferating cells or immortal cells in animals are provided. The inappropriately or pathologically proliferating cells or immortal cells exist and reproduce independently of cells' normal regulatory mechanisms. These cells are pathologic because they  
25 deviate from normal cells as a result of activity of a cellular element, i.e., L1RT. Of course, the inappropriately proliferating cells as used herein may be benign hyperproliferating cells but unless stated otherwise these cells refer to malignant hyperproliferating cells such as cancer cells characteristic of, for example, osteosarcoma, breast carcinoma, ovarian carcinoma, lung carcinoma, adrenocortical  
30 carcinoma or melanoma.

In particular, methods for preventing or treating human tumors characterized as expressing L1RT are provided. The prevention or treatment of the disorders,

according to the present invention, is achieved by the utilization of inhibitors or antagonists of L1RT. The inhibitor(s) or antagonist(s) used in the present invention are those that directly or indirectly interact with L1RT to inhibit its expression (or activity) and/or those that get incorporated into telomere and thus prevent telomere  
5 from further elongation despite the functional L1RT thereby inhibiting the growth of cells expressing L1RT. Thus, the inhibitors or antagonists of L1RT are used for inhibiting the growth of cells. For example, when the inhibitors or antagonists of L1RT are administered to a patient, these cause progressive telomere shortening, cell cycle arrest in the cells and/or massive apoptosis of the cells expressing L1RT. In the  
10 present invention, the terms "inhibiting the growth" or "inhibition of growth" may also mean reducing or preventing cell division. Inhibition of growth of cells expressing L1RT, in the present invention, may be about 100% or less but not 0% . For example, the inhibition may be from about 10% to about 100%, preferably at least about 25%, and more preferably at least about 50%, still more preferably at least  
15 about 90%, 95% or exactly 100% compared to that of the control cells (control cells express L1RT but are not treated with an inhibitor or antagonist). The inhibition of growth can be measured by any methods known in the art. For example, viable cell number in treated samples can be compared with viable cell number in control samples, determined after incubation with vital stains. In addition, growth inhibition  
20 can be measured by assays that can detect reductions in cell proliferation in vitro or in vivo, such as tritiated hydrogen incorporation assays, BdU incorporation assay, MTT assay, changes in ability to form foci, anchorage dependence or losing immortalization, losing tumor specific markers, and/or inability to form or suppress tumors when injected into animal hosts (Dorafshar et al., 2003, J Surg Res.,114:179-  
25 186; Yang et al., 2004, Acta Pharmacol Sin., 25:68-75).

The development of a cancerous tumor from a single immortalized cell or few such cells may take several months to years in humans. By practising the present invention, however, cancer can be prevented because the ability of the tumorigenic ALT cells treated with L1RT inhibitors lose their proliferative potential before they  
30 have had a chance to grow into a tumor. Further, periodic preventative administration of L1RT inhibitors or antagonists to at risk groups in order to stop tumor progression

before clinical manifestation of cancer could potentially decrease the rate of new cancer cases significantly.

The inhibitor or antagonist of the L1RT used in the present invention can be an inorganic compound, an organic compound, an antisense sequence, a double-stranded RNA (dsRNA) corresponding to a defined target region in L1RT mRNA, a  
5 dominant negative mutant of the L1RT protein, an antibody or a small molecule.

In one embodiment of the invention, organic compounds such as, for example, nucleoside analogs are used as inhibitors or antagonists of L1RT. Thus, one of the approaches for targeting L1RT is by administration of nucleoside analog(s) to cancer  
10 patients. The nucleoside analogs can mimic the building blocks used by L1RT to extend the chromosomal ends in telomerase negative cells. These fake building blocks (i.e., nucleoside analogs) that are incorporated into chromosomal ends by L1RT may interfere with the function of the telomeres and thereby contributing to telomere shortening, cell cycle arrest and cell death.

15 There are a number of nucleoside analogs known to one skilled in the art. Indeed, nucleoside analogues are known class of antiretrovirals and a number of nucleoside analog drugs have been approved for the treatment of HIV infected humans. These drugs do stop HIV from multiplying by interfering with copying HIV's genetic material (RNA) into the form of DNA. Examples of nucleoside  
20 analogues that may be used in the present invention are, 3'-azido-2',3'-dideoxythymidine (AZT), 2',3'-dideoxyinosine (ddI), 2',3'-dideoxy-3'-deoxythymidine(d4T), acyclovir, ganciclovir. Precursors (or prodrugs) of these nucleoside analogues (e.g., valganciclovir) can also be used.

Since L1RT is a key factor in cancers of telomerase negative cells, the present  
25 discovery of noncompetitive inhibitors of the activity of this key enzyme represents a potential breakthrough in cancer research and treatment. The demonstration that nucleoside analogs (e.g., AZT and ganciclovir) clearly block ALT cancer in a widely accepted model systems (described below), confirms that the present invention truly represents a dramatic breakthrough. Although not suggesting the advantageous uses  
30 made possible by this invention, the previous administration of AZT to AIDS patients means that AZT can be readily administered to cancer patients.



Indeed, nucleoside analogs have been used to modify telomerase activity in cancer cells to levels close to that found in normal cells as a means for cancer therapy. The concentration of nucleoside analogs required to inhibit L1RT, however, can be several fold lower than that required to inhibit telomerase. For example, the  
5 concentration of AZT required for inhibiting L1RT activity can be orders of magnitude lower (e.g., 10 to 1000 fold lower) than that required for inhibiting telomerase activity. The susceptibility of L1RT to such low levels of nucleoside analogs is quite unexpected and this unexpected finding now offers an advantageous avenue of therapy for treatment of L1RT specific cancers. Importantly, the present  
10 invention provides for the selection of effective doses significantly lower than the levels that may otherwise be used in cancer patients. The studies of this invention indicate that AZT will be useful in cancer at levels that achieve nanomolar drug levels rather than 200  $\mu$ M to 800  $\mu$ M.

Further, the present use of nucleoside analogs to AIDS patients, coupled with  
15 the ability to use significantly lower doses of AZT for HIV therapy, should speed regulatory approval for the use of AZT in the treatment of L1RT induced and/or mediated cancers. Moreover, this invention is not limited to the use of AZT to treat L1RT induced and/or mediated cancers. In fact, the use of L1RT inhibitors is broadly applicable to a range of other disorders in which L1RT is a factor. These include, for  
20 example, L1 induced mutations in the gene for blood factor VIII inducing hemophilia A, in the X-linked retinitis pigmentosa 2, in the dystrophin gene, in the DMD gene resulting in X-linked dilated cardiomyopathy and in the X-linked gene CYBB causing chronic granulomatous disease (Woods-Samuels et al., 1989, Genomics, 4:290-296; Schwahn et al., 1998, Nat. Genet., 19:327-332; Holmes et al., 1994, Nat Genet.,  
25 7:143-148; Yoshida et al., 1998, Hum Mol Genet., 7:1129-1132; Brouha et al., 2002, Am J Hum Genet., 71:327-336).

The nucleoside compounds may be administered either singly or in combinations of different analogs and by any routes of administration, including oral administration. AZT and ganciclovir or its prodrug, valganciclovir, are the preferred  
30 nucleoside analogs. AZT is commercially available and AZT formulations are described in a number of U.S. Patents. See, for example, U.S. Patent 5,683,990. The cells with ALT will be selectively targeted because these cells depend on L1RT for

elongating or maintaining telomeres and the elongation or maintenance of telomeres requires the incorporation of the nucleosides and/or their analogs. To the extent any specific targeting agent is desired for delivering the analogs to exert anti-cancer effects, the use of targeted AZT and/or other analogs are contemplated herein.

- 5 Accordingly, in some embodiments, pharmaceutical compositions may have the active compound, in this case, AZT or another nucleoside analog, which has been conjugated to a targeting agent (e.g., a peptide) for specific delivery to particular target cells or to nuclear portion within cells.

In another aspect of the invention antisense sequence(s), also referred to herein  
10 as antisense oligonucleotide(s) or antisense polynucleotide(s) are used as inhibitors or antagonists of L1RT. The antisense sequences in the present invention are either substantially or fully complementary to a nucleic acid encoding L1RT. The complementarity (whether full or substantial complementarity) of the antisense  
15 sequences is such that they specifically hybridize with the target nucleic acid sequence and interfere with L1RT function, expression or otherwise, and the interference is sufficient to inhibit the growth of the cells.

The nucleic acid encoding L1RT can be DNA, RNA transcribed from such DNA or a cDNA of the RNA. The L1 nucleic acid and amino acid sequences of various mammals, such as mouse, monkey and humans have been sequenced (see  
20 GenBank Accession numbers AY053456, AF036235, AF148856 and GI5070620) (see also, GenBank protein accession AAD39215 for L1RT ORF2 sequence). In the context of the present invention, L1RT mRNA is a preferred nucleic acid for which antisense nucleic acid sequences are designed. For example, a series of antisense phosphorothioate oligonucleotides, 20 or more nucleotides in length, targeting the  
25 nucleic acid encoding L1RT are designed. Generally, the antisense sequences in the present invention may be designed to bind to the promoter or other control regions and coding and/or non-coding regions of L1RT. The antisense sequences preferably target L1RT nucleic acid sequence portion encompassing a start codon. It is also contemplated that the most effective antisense sequences or constructs will include  
30 regions complementary to coding and non-coding regions of L1RT. One can readily test the effectiveness of a given antisense construct simply by testing the construct in vitro to determine whether normal cellular function is affected. It is preferred that the

selected antisense sequence inhibits L1RT activity or expression to the level that is insufficient for inducing or mediating telomere lengthening in ALT cells.

Interference with L1RT expression can happen due to any mechanism. For example, it is believed that such antisense sequences bind to, and interfere with the translation of, the sense L1RT mRNA. Alternatively, the antisense sequence may render the L1RT mRNA susceptible to nuclease or ribozyme digestion, interfere with transcription, or interfere with processing of L1RT mRNA, repress transcription of mRNA from the L1RT gene, or act through some other mechanism, e.g., through ribozymes. Ribozymes, which are well known to those skilled in the art, are molecules of RNA that have catalytic activity. The ribozymes of the invention are antisense sequences that bind and enzymatically cleave and inactivate L1RT RNA. Useful ribozymes can comprise 5'- and 3'-terminal sequences complementary to the L1RT RNA and can be engineered by one of skill on the basis of the L1RT RNA sequence. However, the particular mechanism by which the antisense sequences interfere with L1RT expression is not critical so long as the end result is met.

Generally, to assure specific hybridization, the antisense sequence is substantially complementary to the target L1RT mRNA sequence. In certain embodiments, an antisense sequence that is fully or exactly complementary to the target nucleic acid sequence or two or more antisense sequences fully complementary to different subsequences of a given L1RT target nucleic acid sequence may be used. A Subsequence is a sequence of nucleic acid residues or nucleotides that is a part of a longer sequence of nucleic acid residues such as, for example, an antisense sequence corresponding to nucleotides 1987-2800 of human L1 reprotansposon (GenBank GI: 5070620).

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Table. Exemplary sequences for use in interfering with L1RT mRNA.

SEQ ID NO:	Nucleic Acid Sequence
SEQ ID NO:1 (a sequence antisense to L1RT mRNA results when the sequence set	5'-atga caggatcaac ttcacacata acaatattaa ctttaaatat aaatggacta aattctgcaa ttaaaagaca cagactggca agttggataa agagtcaaga cccatcagtg tgctgtattc aggaaacca tctcacgtgc agagacacac

<p>forth herein, SEQ ID NO:1, in reverse orientation, is expressed in an expression vector)</p>	<p>ataggctcaa aataaaagga  tggaggaaga tctaccaagc caatggaaaa caaaaaaagg  caggggttgc aatcctagtc  tctgataaaa cagactttaa accaacaag atcaaaagag  acaaagaagg ccattacata  atggtaaagg gatcaattca acaagaggag ctaactatcc  taaataattta tgcacccaat  acaggagcac ccagattcat aaagcaagtc ctcaagtacc  tacaagaga cttagactcc  cacacattaa taatgggaga ctttaacacc ccactgtcaa  cattagacag atcaacgaga  cagaaagtca acaaggatac ccaggaattg aactcagtc  tgcaccaagc agacctata  gacatctaca gaactctcca ccccaaatca acagaatata  catttttttc agcaccacac  cacacctatt ccaaaattga ccacatagtt ggaagtaaag  ctctcctcag caaatgtaaa  agaacagaaa ttataacaaa ctatctctca gaccacagtg  caatcaaact agaactcagg  attaagaatc tcaactcaaag ccgctcaact acatggaaac  tgaacaacct gctcctgaat  gactactggg tacataacga aatgaaggca gaaataaaga  tggtctttga aaccaacgag  aacaagaca ccacatacca gaatctctgg gacgcattca  aagcagtgtg tagagggaaa  tttatagcac taaatgccta caagagaaag cagga-3'</p>
<p>SEQ ID NO:2 (a sequense antisense to a portion of L1RT mRNA)</p>	<p>5'-CCA GAG ATT CTG GTA TGT GGT GTC TTT GTT-3'</p>
<p>SEQ ID NO:3 (a</p>	<p>5'-CTT TCT CTT GTA GGC ATT TAG TGC TAT AAA-3'</p>

sequence antisense to a portion of L1RT mRNA)	
SEQ ID NO:4 (a sequence antisense to a portion of L1RT mRNA)	5'-CTC TTG CTT TTC TAG TTC TTT TAA TTG TGA-3'
SEQ ID NO:5 (a sequence antisense to a portion of L1RT mRNA)	5'-CTT CAG TTC TGC TCT GAT TTT AGT TAT TTC-3'
SEQ ID NO:6 (a sequence antisense to a portion of L1RT mRNA)	5'- TCC TGC TTT CTC TTG TAG GCA -3'

The antisense sequences, e.g., DNA, RNA, modified, analogues or the like can be made using any suitable method for producing a nucleic acid, such as the chemical synthesis and recombinant methods disclosed herein (see, examples section) or such methods known to one of skill in the art. In one embodiment, for example, antisense RNA molecules of the invention may be prepared by de novo chemical synthesis or by cloning. For example, an antisense RNA that hybridizes to L1RT mRNA can be made by inserting (ligating) a sequence set forth in SEQ ID NO:1 in reverse orientation, operably linking it to a promoter and expressing it in an expression vector (e.g., plasmid). Provided that the promoter and, preferably termination and polyadenylation signals, are properly positioned, the strand of the inserted sequence corresponding to the noncoding strand will be transcribed and act as an antisense sequence of the present invention.

In some embodiments, the antisense sequences may also include modified antisense nucleic acid sequences having nucleotide additions, substitutions, deletions or modifications, or other nucleic acid sequences or non-nucleic acid moieties so long

as specific binding to the relevant target sequence, i.e., L1RT RNA or its gene/cDNA, is retained as a functional property of the sequences.

For example, a modified antisense nucleic acid sequence consisting of the nucleotides identical to that set forth in SEQ ID NO: 2, 3, 4, 5 or 6 except that, over  
5 the entire length corresponding to the nucleotide sequence of SEQ ID NO: 2, 3, 4, 5 or 6, the modified antisense nucleic acid sequence has one or more nucleotide substitutions, deletions or insertions. Identity or similarity, as known in the art, is a relationship between two or more polynucleotide sequences as determined by comparing the sequences. Identity also means the degree of sequence relatedness between  
10 polynucleotide sequences, as determined by the match between strings of such sequences from 5' to 3' end for polynucleotides. "Identity" can be readily calculated by art known methods. *See e.g., Altschul et al., Nucleic Acids Res., 25:3389-3402 (1997).* For example, sequence identity may be optimized by alignment algorithms known in the art and calculating the percent difference between the nucleotide  
15 sequences. Effective antisense sequences can be determined by using, for example, GCG (Genetics Computer Group, Madison Wis.) or combinatorial arrays of oligonucleotides or DNA microarrays, which techniques are known to one skilled in the art.

In the present invention, L1RT antisense polynucleotides, RNA, DNA or  
20 modified nucleic acid that can be produced by direct chemical synthesis may also be used. Chemical synthesis is generally preferred for the production of oligonucleotides or for oligonucleotides and polynucleotides containing nonstandard nucleotides (e.g., probes, primers and antisense oligonucleotides) for use in the present invention. Direct chemical synthesis of nucleic acids can be carried out by procedures known in  
25 the art. One of ordinary skill in the art will recognize that while chemical synthesis of DNA may often be limited to sequences of about 100 or 150 bases, longer sequences may be obtained by the ligation of shorter sequences or by more elaborate synthetic methods. It will be appreciated that the L1RT antisense oligonucleotides of the invention can be made using nonstandard bases or nonstandard backbone structures to  
30 provide desirable properties such as, for example, increased nuclease-resistance, tighter-binding, stability or a desired  $T_m$ ).

A wide variety of useful modified oligonucleotides may be produced, including peptide nucleic acid (PNA). Peptide nucleic acid is an analogue of DNA in which the backbone is a pseudopeptide (an amide, in particular N-ethylaminoglycine backbone) rather than a sugar (see, Peter E. Nielsen (Ed), Peptide Nucleic Acids: 5 Protocols and Applications, First Edition, 1999, Horizon Scientific Press). Such a backbone has been reported to result in stronger binding and greater specificity than normally achieved. In addition, the unique chemical, physical and biological properties of PNA have been exploited to produce powerful biomolecular tools, antisense and antigene agents, molecular probes and biosensors. Further teaching of 10 PNA compounds can be found in U.S. Pat. Nos. 5,539,082; 5,714,331 and 5,719,262.

In some embodiments, chimeric oligonucleotides, triplex-forming antisense sequences, RNA-DNA oligonucleotides (RDO), oligonucleotides having backbone analogues, such as phosphodiester, phosphorothioate, phosphorodithioate and such others known in the art may be synthesized and used. For example, a series of 15 antisense phosphorothioate oligonucleotides, 30 nucleotides in length, targeting a nucleic acid encoding L1RT may be used.

It is often useful to label the antisense polynucleotides of the invention, for example, when the L1RT polynucleotides are to be used for detection of L1RT expression, or for the diagnosis and prognosis of conditions related to the 20 inappropriate hyperproliferation. The labels may be incorporated by any of a number of means well known to those of skill in the art. Suitable labels are any composition detectable by photochemical, biochemical, immunochemical, chemical, or spectroscopic means. For example, useful labels include  $^{32}\text{P}$ ,  $^{35}\text{S}$ , fluorescent dyes, enzymes (e.g., as commonly used in an ELISA), biotin-streptavidin, digoxigenin, 25 haptens and proteins for which antisera or monoclonal antibodies are available, or nucleic acid molecules with a sequence complementary to a target. The label often generates a measurable signal, such as radioactivity, that can be used to quantitate the amount of bound detectable moiety.

In another aspect of the invention double-stranded RNAs (dsRNAs) 30 corresponding to a defined target region in L1RT mRNA, are used as inhibitors or antagonists of L1RT. The dsRNAs induce RNA-targeted gene-silencing of L1RT which result in reduction or loss of L1RT expression in targeted cells. RNA-targeted

gene silencing is well known to one of ordinary skill in the art (Ahlquist, 2002, Science, 296:1270-1273). The dsRNAs may be endogenously synthesized or exogenously applied but only catalytic amounts of dsRNA are required to induce the silencing. A nucleotide sequence from a portion of the L1RT gene is chosen to  
5 produce inhibitory RNA, which may be partially or fully double-stranded type. The inhibition is specific because a nucleotide sequence from a portion of the target gene is chosen to produce inhibitory RNA.

There are different methods known in the art to induce RNA-targeted gene-silencing such as small interfering RNA, short hairpin RNA, expressed long  
10 interfering RNA, expressed short interfering RNA and expressed short hairpin RNA. It is preferred that certain dsRNAs (small interfering RNA and short hairpin RNA) include modifications to either the phosphate-sugar backbone or the nucleoside. The RNA duplex formation may be initiated either inside or outside the cell. The RNA may be introduced in an amount which allows delivery of at least one copy per cell  
15 with higher doses of double-stranded material may yield more effective inhibition. Inhibition is sequence-specific in that L1RT mRNA nucleotide sequences corresponding to the duplex region of the RNA are targeted for genetic inhibition. RNA sequences with insertions, deletions, and single point mutations relative to the target sequence can also be found to be effective for inhibition.

20 The RNA may be delivered to cells or directly introduced into intercellular spaces of a tissue or into the vascular system of an organism. It may also be delivered orally to the patients. Methods for oral introduction include direct mixing of dsRNA with food of the patient, as well as engineered approaches in which a species that is used as food is engineered to express the RNA, then fed to the organism to be  
25 affected. Physical methods of introducing nucleic acids include injection directly into the cell or extracellular injection into the patient of an RNA solution.

In another aspect, dominant negative mutants of the L1RT protein or nucleic acids having a sequence encoding a dominant negative mutant L1RT protein or non-functional fragment or derivative thereof are administered to inhibit L1RT function by  
30 interfering with the interactions of L1RT and with other molecules in the cell. It is believed that the L1RT must directly interact with a portion of the telomere for telomere elongation.



Therefore, L1RT mutants that are defective in function but effective in binding to the portion of the telomere can be used as a dominant negative mutant to compete with the wild type L1RT. Dominant non-functional L1RT can be engineered for expression in cancer cells that inappropriately overexpress L1RT. Given that the protein and nucleic acid sequences of the wild type L1RT is known, one skilled in the art can create dominant negative mutants of L1RT suitable for use in the present invention. Such dominant negative mutants may be administered to cells in vivo or in vitro according to the standard delivery methods already known in the art. In a preferred aspect of the invention, the therapeutic nucleic acid has an L1RT nucleic acid that is part of an expression vector that expresses a dominant non-functional L1RT protein or fragment or chimeric protein thereof in cancer cells.

In another aspect of the present invention, antibodies or binding portions thereof specific to L1RT are used as inhibitors or antagonists of L1RT. Specifically, the present invention contemplates the prevention and treatment of L1RT induced cancer in humans as well as other animals through the use of antibodies to L1RT. Both polyclonal and monoclonal antibodies and binding portions (Fab fragments and Fv fragments) of such antibodies are contemplated in the context of the present invention. Such antibodies may be made in a variety of animals including, mice, rabbits, monkeys, chimpanzees, cows (e.g., in the milk) and birds. The present invention also contemplates human and humanized antibodies. The antibodies can be used preventively or during the acute stage of pathological cell proliferation.

In one embodiment, the present invention contemplates a method in which the antibodies which bind to L1RT protein are administered so that the antibodies react with L1RT. In another embodiment, the antibodies are combined with other reagents including but not limited to other antibodies. The administration of antibodies can be carried out orally, parenterally or by other suitable routes.

The antibody production may be effected by techniques which are well-known in the art. For example, mammalian lymphocytes are immunized by in vivo immunization of the animal (e.g., a mouse) with human L1RT protein or polypeptide. Such immunizations are repeated as necessary at intervals of up to several weeks to obtain a sufficient titer of antibodies. Hybridomas may be produced and cultured, and the resulting colonies are screened for the production of the desired monoclonal

antibodies. Colonies producing such antibodies are cloned, and grown either in vivo or in vitro to produce large quantities of antibody.

As discussed further in detail below, the efficacy of various inhibitors or antagonists may be shown in standard experimental animal models prior to  
5 administration to subjects or patients. The subject, or patient, to be treated using the methods of the invention is preferably human, and can be a fetus, child, or adult. Other mammals that may be treated can be mice, rats, rabbits, monkeys and pigs.

The inhibitors or antagonists can be used alone or in combination with other chemotherapeutics or otherwise. For example, therapy of L1RT induced cancers may  
10 be combined with chemo and/or radiotherapy to treat cancers induced by telomerase or some other factors. Examples of chemotherapeutic agents known to one skilled in the art include, but are not limited to, anticancer drugs such as bleomycin, mitomycin, nitrogen mustard, chlorambucil, 5-fluorouracil (5-FU), floxuridine (5-FUdR), methotrexate (MTX), colchicine and diethylstilbestrol (DES). To practice combined  
15 therapy, one would simply administer to an animal an inhibitor component of the present invention in combination with another anti-cancer agent in a manner effective to result in their combined anti-cancer actions within the animal or patient. The agents would therefore be provided in amounts effective and for periods of time effective to result in their combined presence in the region of target cells. To achieve this goal,  
20 the agents may be administered simultaneously, either in a single composition, or as two distinct compositions using different administration routes. Alternatively, the two treatments may precede, or follow, each other by, e.g., intervals ranging from minutes to hours or days. By way of example, and not limitation, the average daily doses of AZT for systemic use may be 10 mg/kg per day for human adults, 20 mg/kg per day  
25 for mice.

Some variation in dosage may occur depending on the condition of the subject being treated. The physician responsible for administration will be able to determine the appropriate dose for the individual patient and may depend on multiple factors, such as, the age, condition, file history, etc., of the patient in question.

30 Accordingly, the methods of the invention can be used in therapeutic applications for conditions and diseases associated with L1RT induced pathological proliferation of cells. Diseases that would benefit from the therapeutic applications of

this invention include all diseases characterized by cell hyperproliferation including, for example, solid tumors and leukemias, and non-cancer conditions. It is further contemplated that the method of the invention can be used to inhibit the growth of cancer cells not only in an *in vivo* context but also in an *ex vivo* situation. The method  
5 of the invention is particularly useful for inhibiting the growth of pathologically proliferating human cells *ex vivo*, including, but not limited to, human cancer cells - osteosarcoma, breast carcinoma, ovarian carcinoma, lung carcinoma, adrenocortical carcinoma or melanoma.

The present invention provides methods and kits for identifying  
10 inappropriately, pathologically or abnormally proliferating cells due to the expression of L1RT in the cells. The methods can be used as a screening method that aids in diagnosing the presence of a cancerous cell or tumor in a patient by determining the presence (and/or level) of expression of L1RT in tissue from the patient, the presence of L1RT expression being indicative of cancer cells or pathological cell proliferation  
15 in the patient.

For example, cancerous tumor samples can be diagnosed by the detection of L1 specific mRNA expression measured by a variety of methods including, but not limited to, hybridization using nucleic acid, Northern blotting, *in situ* hybridization or RNA microarrays, or the presence of L1 retrotransposon ORF1 and/or ORF2 encoded  
20 proteins measured by variety of methods including, but not limited to, Western blotting, immunoprecipitation or immunohistochemistry, or enzymatic activity of reverse transcriptase.

Cancer cells showing ALT can also be diagnosed by determining the absence of catalytic subunit mRNA expression (measured by a variety of methods including,  
25 but not limited to, Northern blotting, RNA protection assay, *in situ* hybridization, RT-PCR, real time RT-PCR or RNA microarrays), or the absence of telomerase catalytic subunit translation (measured by a variety of methods including, but not limited to, Western blotting, immunoprecipitation or immunohistochemistry). Another characteristic of cells showing ALT is the presence of long and heterogeneous  
30 telomeres (Bryan et al., 1997, Nature Medicine, 3:1271-1274). Accordingly, a diagnostic method may include detection of the presence of long and heterogeneous telomeres as an indicator of cells with ALT. The method includes, but is not limited

to, terminal restriction digest and its modification, *in situ* hybridization with a telomere specific probe or flow cytometry with telomere specific DNA or PNA probes.

In a preferred embodiment, nucleic acid probes directed against L1RT can be used to detect presence and/or increases in L1RT mRNA levels in tissues undergoing rapid proliferation, such as primary cancer cells, including human osteosarcoma, breast carcinoma, ovarian carcinoma, lung carcinoma, adrenocortical carcinoma or melanoma. Thus, the present invention provides methods of using nucleic acid probes that are complementary to a subsequence of an L1RT to detect and identify pathologically proliferating cells, including cancer cells. For example, the method for identifying a pathologically proliferating cell may involve using a nucleic acid probe directed against an L1RT mRNA to compare the level of expression of L1RT mRNA in a test cell with the level of expression of L1RT mRNA in a control cell. A test cell is identified as a pathologically proliferating cell when the level of L1RT expression is observed as in the control cell.

It is preferred that the nucleic acid probe used in the method of the present invention is fully complementary to a human L1RT nucleic acid sequence, preferably mRNA, and the test cell is a human cell. An example of nucleic acid probe that is fully complementary to a human L1RT RNA sequence is 5'- TCC TGC TTT CTC TTG TAG GCA -3' (SEQ ID NO:6). The nucleic acid probe used in the method of the invention, however, may also be substantially complementary to an L1RT mRNA or an L1RT retrotransposon RT sequence of human mouse or other mammal. It will be apparent to one of ordinary skill in the art that substitutions may be made in the nucleic acid probe which will not affect the ability of the probe to effectively detect the L1RT RNA in pathologically proliferating cells (e.g., cancer cells) and thus, such substitutions are within the scope of the present invention. The nucleic acid probe used in the method of the present invention can be a DNA probe, or a modified probe such a peptide nucleic acid probe, a phosphorothioate probe, or a 2'-O methyl probe. The length of the nucleic acid probe may be from about 8 or 10 to 50 nucleotides, preferably from about 15 to 25 nucleotides in length. The method of the invention can be readily performed in a cell extract, cultured cell, or tissue sample from a human, a mammal, or other vertebrate.

The methods of the present invention are useful for detecting the inappropriately, pathologically or abnormally proliferating cells due to the expression of L1RT in the cells in vitro, in cell cultures, and in human cells and tissues, such as solid tumors and cancers (e.g., human osteosarcoma, breast carcinoma, ovarian  
5 carcinoma, lung carcinoma, adrenocortical carcinoma or melanoma).

The present invention also provides kits for detecting and/or inhibiting hyperproliferating cells or cancer cells. The kit can have a nucleic acid probe that is fully or substantially complementary to a subsequence of an L1RT mRNA. The kits for inhibiting the proliferation of pathologically proliferating cells, the kit comprising  
10 the step of contacting the cells with may have an agent, e.g., an antisense oligonucleotide that is substantially complementary, preferably fully complementary, to a subsequence of an L1RT nucleic acid, which agent upon contacting the cells can affect pathological proliferation. The kits can be in the form of a container containing one or more of the above-discussed nucleic acid probes, antisense oligonucleotides, or  
15 other suitable agents with or without detection labels discussed herein. The kits may contain a suitable membrane for separation and hybridization of sample RNA, DNA or protein, preferably in the form of an assay apparatus that is adapted to use with the claimed methods. The kits can also include instruction manuals for carrying out the methods of the present invention. The kits may also include reagents useful for  
20 detecting the presence of the detectable labels and/or materials useful in the performance of various assays including positive, negative controls, internal and/or external controls. Exemplary reagents and materials are RNA extraction buffers, hybridization buffers, test tubes, transfer pipettes, and the like.

The inhibitors or antagonists of the L1RT that can be used in methods of the  
25 present invention should not be limited in any way to the specific compounds mentioned in the present application. Given that the present invention discloses a target responsible for hyperproliferation of cells, a number of other useful inhibitors or antagonists of the L1RT can be identified by simple screening methods. The active compounds may include fragments or parts of naturally-occurring or prior art  
30 compounds. However, prior to testing of such compounds in humans, it may be necessary to test a variety of candidate agents in screening assays to determine which have potential as anti-tumor drugs. A number of assays are known in the art for

determining the effect of a drug on cancer. Therefore, in particular embodiments, the present invention concerns a method for identifying or selecting compounds that will modulate expression or activity of L1RT. Drugs which interfere with the biological activity of L1RT are good candidates for anti-tumor drugs, because they affect one of  
5 the steps that leads to uncontrolled proliferation or a continuous increase in cell number.

Screening for compounds or drugs may be performed using purified L1RT enzyme, an in vitro model, cell cultures, a genetically altered cell or animal, or xenograft model antitumor assays. Of particular interest are screening assays for  
10 agents that have a low toxicity for human cells. Candidate agents encompass numerous chemical classes, though typically they are organic molecules, antisense polynucleic acids or small organic compounds having a molecular weight of more than 50 and less than about 2,500 daltons, analogs of purines and pyrimidines or combinations thereof. Known pharmacological antitumor agents may be subjected to  
15 further chemical modifications, such as amidification, to produce structural analogs. If the screening assay is a binding assay, one or more of the molecules may be joined to a label, where the label can directly or indirectly provide a detectable signal. Various labels such as radioisotopes, fluorochromes, chemiluminescent agents, enzymes and specific binding molecules, particles, e.g. magnetic particles may be  
20 used. A variety other reagents like salts, neutral proteins, e.g. albumin, detergents, etc may also be used to facilitate optimal protein-protein binding and/or reduce non-specific or background interactions. Reagents that improve the efficiency of the assay, such as protease inhibitors, nuclease inhibitors, anti-microbial agents may be used.

For example, a screening assay or a method for identifying a compound or  
25 agent in its simplest form may include incubating a candidate compound or compounds to be tested with a cell expressing L1RT under conditions in which, but for the presence of the compound or compounds to be tested, the interaction of L1RT and other cell components induces a detectable or measurable biological effect or a chemical effect (example addition of nucleotides or analogs to the telomere or  
30 maintenance of telomere length) and then determining the ability of L1RT to interact with the cell components to induce the detectable or measurable biological effect or the chemical effect in the presence of the compound or compounds to be tested. If the

candidate compound or the tested compound modulates the interaction L1RT with other cell components, then that compound is selected.

Other assays of interest can be, for example, a cell line expressing L1RT or an expression construct having an L1RT gene may be introduced into a cell line under conditions that allow L1RT expression. To this cell line candidate agent(s) is(are)  
5 added, and the ability to inhibit or down-regulate L1RT activity is detected. The level of L1RT activity may be determined by a functional readout or assay including alterations in L1RT expression levels, binding or inhibition of binding to a telomere or some other substrate, apoptosis, presence or lack of growth, presence or lack of  
10 metastasis, presence or lack of cell division, presence or lack of cell migration, presence or lack of soft agar colony formation, presence or lack of contact inhibition, presence or lack of invasiveness, and/or presence or lack of tumor progression or other malignant phenotype.

For example, a method for determining the ability of a candidate compound to  
15 decrease the wild-type L1RT expression in cells and to concomitantly induce apoptosis in those cells may be carried out by obtaining a cell expressing L1RT, admixing a candidate substance with the cell; and determining the ability of the candidate substance to reduce the L1RT content and/or telomere length on the chromosomes of the cell.

20 Another simple example to identify a candidate substance as being capable of interfering with L1RT expression can be as follows: one may measure or determine the L1RT status of a cell. If that cell has the ability to express L1RT, its basal L1RT content in the absence of the added candidate compound is measured. One may then add the candidate compound to the cell and re-determine the wild-type L1RT  
25 expression in the presence of the candidate compound. A candidate compound that decreases the L1RT expression relative to the cell's L1RT expression in the absence of the test or candidate compound is indicative of a candidate compound with wild-type L1RT expression inhibiting capability. It can, therefore, have prophylactic and therapeutic cancer reducing and apoptotic potential.

30 The present invention also encompasses the use of various animal models. By developing or isolating cell lines that express L1RT one can generate disease models in various laboratory animals. These models may employ the subcutaneous,

orthotopic or systemic administration of cells to mimic various disease states. For example, the IICF/c fibroblast cell line (ALT) can be transfected with pSV2neo-EJras plasmid DNA (containing the activated c-Ha-ras oncogene from the EJ bladder carcinoma cell line), selected with G418, and injected subcutaneously into nude mice to obtain ALT tumors. The resulting tumors do not show any detectable telomerase activity in telomeric repeat amplification protocol (TRAP) assay, and Southern analysis shows that they retained the TRF length pattern diagnostic of ALT (Yeager et al., Cancer Res. 1999, 59(17):4175-9). Finally, telomerase knock out animals (e.g., telomerase KO mice -/-; Rudolph et al., 1999, Cell, 96:701-712) or transgenic animals that express a wild-type L1RT as a transgene in the animals may be utilized as models for treatment. Of course, animal models provide a useful vehicle for testing combinations of agents as well. Determining the effectiveness of a compound in vivo may involve a variety of different criteria including, but are not limited to, survival, tumor regression, arrest or slowing of tumor progression, elimination of tumors and inhibition or prevention of metastasis.

Treatment of animals with test compounds will involve the administration of the compound, in an appropriate form, to the animal. Administration will be by any route that could be utilized for clinical or non-clinical purposes, including but not limited to oral, nasal, buccal, rectal, vaginal or topical. Alternatively, administration may be by intratracheal instillation, bronchial instillation, intradermal, subcutaneous, intramuscular, intraperitoneal or intravenous injection. Specifically contemplated are systemic intravenous injection, regional administration via blood or lymph supply and intratumoral injection.

Of course, the screen may include appropriate control values (e.g., the level of L1RT expression or production in isolated cells or animals showing ALT in the absence of candidate compound(s)). Test compounds or candidate compounds which are considered positive, i.e., likely to be beneficial in the treatment of cancer will be those which have a substantial growth inhibitory effects (e.g., test agents that are able to reduce the growth of cells preferably by at least 20% more preferably by at least 50%, and most preferably by at least 80%, still more preferably by about 90 to 100%.

Such compounds would be important in a number of aspects. They would be important in regimens for the treatment of L1RT-related cancers, whether



administered alone or in combination with chemo- and radiotherapeutic regimens known to one skilled in the art in the treatment of cancer. Alternatively, by simply reducing L1RT, these compounds will be instrumental in selectively inducing massive apoptosis of cancer cells.

5           The compounds having the desired pharmacological activity are selected and may be administered in a physiologically or pharmaceutically acceptable carrier to a host for treatment of proliferative diseases, etc. Pharmaceutically acceptable carriers are determined in part by the particular composition being administered (e.g., nucleic acid, protein, organic compound, a vector or transduced cell), as well as by the  
10       particular method used to administer the composition. Accordingly, there are a wide variety of suitable formulations of pharmaceutical compositions of the present invention.

          A pharmaceutical composition in the present invention may contain recombinant products. For example, the antisense oligonucleotides or dsRNA  
15       targeted to L1RT can be inserted into any of a number of well-known vectors for the transfection of target cells and organisms. For example, nucleic acids are delivered as DNA plasmids, naked nucleic acid, and nucleic acid complexed with a delivery vehicle such as a liposome. Viral vector delivery systems include DNA and RNA viruses (Porter, 2004, Retroviral vectors for suicide gene therapy, *Methods Mol Med.*,  
20       90:91-106; Wang et al., 2004, Prolonged and inducible transgene expression in the liver using gutless adenovirus: A potential therapy for liver cancer, *Gastroenterology*, 126:278-289). In a specific embodiment, a viral vector that contains an antisense L1RT nucleic acid is used. For example, a retroviral vector or adenoviral vector known in the art for cancer gene therapy can be used. The antisense L1RT nucleic  
25       acid to be used in gene therapy is cloned into a suitable vector, which facilitates delivery of the gene into a patient.

          Methods of non-viral delivery of nucleic acids may include naked polynucleotide, agent-enhanced uptake of polynucleotide, microinjection, particle bombardment, liposomes, immunoliposomes, polycation or lipid:nucleic acid  
30       conjugates. Delivery can be to cells (ex vivo administration) or target tissues (in vivo administration) (Narayanan, Antisense therapy of cancer, *In Vivo*. 1994, 8(5):787-793; Zhang et al., Anti-oncogene and tumor suppressor gene therapy--examples from

a lung cancer animal model, In Vivo. 1994, 8(5):755-769. In a particular embodiment, a nucleic acid molecule is used in which the antisense L1RT sequences and any other desired sequences are flanked by regions that promote homologous recombination at a desired site in the genome, thus providing for intrachromosomal  
5 expression of the antisense L1RT nucleic acid. An example of the sequences that flank 5' end of L1 RT ORF (ORF2) 5'-agaccat caagactagg aagaaactgc atcaactaat gagcaaaatc accagctaac atcata - 3' (SEQ ID NO:7).

The pharmaceutical compositions, inhibitory or antagonistic agents of the present invention can be administered in a variety of ways including orally, topically,  
10 parenterally e.g. subcutaneously, intraperitoneally, by viral infection, intravascularly, etc. Depending upon the manner of introduction, the compounds may be formulated in a variety of ways. Formulations suitable for oral administration can be liquid solutions. Formulations suitable for parenteral administration (e.g., by intraarticular, intraventricular, intranasal, intravenous, intramuscular, intradermal, intraperitoneal,  
15 and subcutaneous routes) include aqueous and non-aqueous, isotonic sterile injection solutions. In the practice of this invention, compositions can be administered, for example, by intravenous infusion, orally, topically, parenterally or intraperitoneally. Oral and parenteral administrations are the preferred methods of administration. Techniques for formulation and administration are routine in the art and further details  
20 may be found, for example, in "Remington's Pharmaceutical Sciences (2000), Gennaro AR(ed), 20th edition, Maack Publishing Company, Easton, PA.

A pharmaceutical composition containing a compound or compounds (e.g., nucleic acid, protein, organic compound, a vector and a transduced cell) for modulating L1RT is administered to a patient in need of the composition in an  
25 effective amount to achieve the intended purpose. Therapeutically effective amount or pharmacologically effective amount are well recognized phrases in the art and refer to that amount of an agent effective to produce the intended pharmacological result. For example, a therapeutically effective amount is an amount sufficient to effect a beneficial therapeutic response in the patient over time (i.e., to treat a disease or  
30 condition or ameliorate the symptoms of the disease being treated in the patient). The amount actually administered will be dependent upon the individual to which treatment is to be applied, and will preferably be an optimized amount such that the

desired effect is achieved without significant side effects. As described further in detail below, the dose may also be determined by the efficacy of the particular inhibitor or antagonistic agent employed and the condition of the patient, as well as the body weight or surface area of the patient to be treated. The size of the dose also  
5 will be determined by the existence, nature, and extent of any adverse side-effects that accompany the administration of , for example, a particular agent, vector or transduced cell type to a particular patient.

Therapeutically effective doses of agent(s) capable of preventing, inhibiting or reducing the incidence of ALT mediated cancer are readily determinable using data  
10 from cell culture assays disclosed herein and/or from in vivo assays using an animal model. In this regard any animal model for L1RT induced cancer known in the art can be used (Hahn et al., 1999, Nature Medicine, 5(10):1164 – 1170; Yeager et al., 1999, Cancer Research, 59(17): 4175-4179). The animal model can also be used to estimate appropriate dosage ranges and routes of administration in humans.  
15 Experimental animals bearing solid tumors of human origin (or art-accepted animal models) are frequently used to optimize appropriate therapeutic doses prior to translating to a clinical environment. Such models are known to be very reliable in predicting effective anti-cancer strategies. For example, mice bearing solid tumors or art-accepted mouse models are widely used in pre-clinical testing to determine  
20 working ranges of therapeutic agents that give beneficial anti-tumor effects with minimal toxicity. Due to the safety already demonstrated in art-accepted models, at least with respect to nucleoside analogs used in the context of AIDS and telomerase-mediated cancer, pre-clinical testing of the present invention will be more of a matter of routine experimentation. In vivo efficacy may be predicted using assays that  
25 measure inhibition of tumor formation (progression), tumor regression or metastasis, and the like.

Exemplary in vivo assays of anti-tumor efficacy of AZT using nude mice subcutaneous (s.c.) tumors grown from the U-2 OS human osteosarcoma cell lines (i.e., xenografts bearing mice) as models are described below.

30 Human cancerous cells needed for in vivo assays may be prepared, for example, as follows: Telomerase- negative, but ALT positive, U-2 OS human osteosarcoma cell lines are obtained from public sources such as ATCC. Cells are

maintained in McCoy 5 media supplemented with 10% fetal calf serum at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. A preliminary test showed that the U-2 OS tumor model required an oncogene expression for significant antitumor activity.

Accordingly, activated *ras*-oncogene expression vector is introduced in near-

5 confluent U-2 OS cells by Lipofectamine 2000™ transfection according manufacturer's instructions. One day before transfection U-2 Os cells are seeded at a density  $1 \times 10^5$  cells in a 6 well-plate. Plasmid pBABE-puro-*ras*-V12 (available publicly) is linearized by restriction digest with Sca I enzyme. The cells are transfected with the linearized construct and grown in culture. One day after the  
10 transfection, cells may be diluted. Then the cells are selected with puromycin ( $0.5 \text{ mg/ml}^{-1}$ ) for 8 days. Another example of human ALT cell line that will be tumorigenic in mouse model is IICF/c fibroblast cell line that is transfected with pSV2neo-EJras plasmid (containing the activated c-Ha-ras oncogene from the EJ bladder carcinoma cell line) DNA, and selected with G418.

15 For in vivo assay, immunodeficient mice, e.g., Swiss homozygous nude (*nu/nu*) mice (or immunodeficient mice, Balb/c-ByJ-Hfh11nu) of about 5-7 weeks old are obtained and maintained in pathogen-free conditions prior to the administration of cancerous cells. Approximately,  $5 \times 10^5$  U-2 OS/*ras*-V12 cells contained in 200 µl of serum-free media are delivered to all animals, briefly  
20 anaesthetized with Metofane, by subcutaneous (s.c.) injection in flank for generating tumors. Alternatively, tumorigenicity could be achieved after subcutaneous injection of about  $30 \times 10^6$  untransformed U-2 OS cells (Manara et al., 2000, Reversal of malignant phenotype in human osteosarcoma cells transduced with the alkaline phosphatase gene, *Bone* 26(3): 215-220). Then the mice are divided into  
25 experimental group and control group.

In one embodiment, impairment of s.c. tumor growth or time to progression rather than decrease in size of an established tumor is assessed. In this embodiment, starting from the day zero, mice in the experimental group receive, for example, AZT (Retrovirtm IV, GlaxoSmithKline) in drinking water. Concentration of AZT in water  
30 can be 2 mg/ml. Fresh solution of AZT is supplied every 3 days. Mice in the control group receive only drinking water. Tumors are measured every 2-3 days. Mice are sacrificed when tumors exceed  $1 \text{ cm}^3$ . Tumor volume is calculated with formula

$4/3\pi r^3$ , where  $r$  is the radius of the tumor. All mice in the control group should develop tumors and all mice in the experimental group remain tumor free.

In another embodiment, the reagents and methods of the invention can be used to promote tumor regression in vivo in animals carrying pre-established tumors; i.e., the reagents of the invention can be used to treat animals with pre-existing tumors. In this case, the cancerous cells are injected subcutaneously in the flank of the nude (*nu/nu*) mice to establish tumors. Once tumors are established after tumor cell implantation, the mice in the experimental group are administered with a composition containing a nucleoside analog effective against L1RT activity, and the mice in the control group receive the same composition but without the nucleoside analogue (e.g., water or saline) 2-3 times daily. Tumor growth is monitored every 2-3 days. When the nucleoside analogue is administered 10-14 days post tumor cell implantation to these tumor bearing animals, retarded tumor growth is observed. Such inhibition of tumor cell growth is not observed in the control group. Few weeks after tumor implantation, only the animals treated with the nucleoside analogue show 100% survival.

For example, xenograft tumors can be subcutaneously generated in immunodeficient mice by the injection of the transformed IIIICF/c fibroblast cells. About  $2 \times 10^6$  cells may be injected subcutaneously into the mice briefly anaesthetized with Metofane. Preferably, the cells are injected along their dorsal flanks. The growing tumors may be measured every 2-3 days. Tumor growth can be followed by measuring with a caliper the longest axis of the tumor and the axis perpendicular to this. Tumor volume may be calculated using the formula  $4/3\pi r^3$ , where  $r$  is the radius of the tumor. The tumors may be excised and weighed prior to processing. Tissues to be used for molecular biological analysis may be snap frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$ . The xenograft tumors will have no detectable telomerase activity in the Telomeric Repeat Amplification Protocol (TRAP) assay. The TRF length pattern diagnostic of cells showing ALT may be verified by Southern analysis.

After induction of the tumors, mice in the experimental groups may be treated with AZT. Mice may be injected i.p. twice a day with solution of AZT in PBS with a total daily dose of 10 mg/kg. Mice in the control group may be injected with PBS.

Alternatively, AZT at the same daily dose may be given in drinking water. Mice in the control group will bear the actively growing tumors and none of the mice in experimental groups will have tumors. As a separate set of controls, telomerase-positive tumors in nude mouse may be induced by injecting the immunodeficient mice with WM1175 (malignant melanoma) or HUT292DM (lung cancer) cells instead of the transformed IIIcF/c fibroblast cells. It should be noted that the telomerase-positive tumors in the immunodeficient mice cannot be inhibited by the AZT at the dose used for inhibiting the growth of the ALT cancer cells.

In another embodiment, in vivo assays that qualify the promotion of apoptosis may also be used. In this embodiment, xenograft bearing mice treated with the therapeutic composition may be examined for the presence of apoptotic foci and compared to untreated control xenograft-bearing mice. The extent to which apoptotic foci are found in the tumors of the treated mice provides an indication of the therapeutic efficacy of the composition.

In designing appropriate doses of agent(s) for the treatment of human ALT-mediated cancers (both early stage tumors and vascularized tumors), one may readily extrapolate from the animal studies described herein in order to arrive at appropriate doses for clinical administration. To achieve this conversion, one would account for the mass of the agents administered per unit mass of the experimental animal and, preferably, account for the differences in the body surface area between the experimental animal and the human patient. All such calculations are well known and routine to those of ordinary skill in the art. Thus, the determination of a therapeutically effective dose is well within the capability of those skilled in the art.

For example, in taking the successful doses of AZT in cell culture assays and in the mouse studies, and applying standard calculations based upon mass and surface area, effective doses for use in human patients would be between about 100 mg and about 1000 mgs AZT per patient per day, and preferably, between about 500 mgs and about 600 mgs AZT per patient per day. Accordingly, using this information, it is contemplated herein that low doses of therapeutic agents (e.g., nucleoside analogs 3'-azido-2',3'-dideoxythymidine (AZT), 2',3'-dideoxyinosine (ddI) or 2',3'-dideoxy-3'-deoxythymidine (d4T) or ganciclovir) for human administration may be about 1, 5, 10, 20, 25 or about 30 mgs or so per patient per day; and useful high doses of

therapeutic agent for human administration may be about 250, 300, 400, 450, 500, 1000, 3000 or about 6000 mgs or so per patient per day. Useful intermediate doses may be in the range from about 500 to about 3000 mgs or so per patient.

Notwithstanding these stated ranges, it will be understood that, given the parameters  
5 and detailed guidance presented herein, further variations in the active or optimal ranges will be encompassed within the present invention. The intention of the therapeutic regimens of the present invention is generally to produce significant anti-tumor effects whilst still keeping the dose below the levels associated with unacceptable toxicity. In addition to varying the dose itself, the administration  
10 regimen can also be adapted to optimize the treatment strategy. A currently preferred treatment strategy is to administer between about 1-500 mgs, and preferably, between about 10-100 mgs of the inhibitor or antagonist of L1RT or therapeutic cocktail containing such, daily within about a 40 days period. Administration can be accomplished via single or divided doses taken orally or, for example, by  
15 administration to the site of a solid tumor directly or in a slow release formulation. The physician responsible for administration will, in light of the present disclosure, be able to determine the appropriate dose for the individual subject, the form and route of administration. Such optimization and adjustment are routinely carried out in the art and by no means reflect an undue amount of experimentation. In administering the  
20 particular doses themselves, one would preferably provide a pharmaceutically acceptable composition according to regulatory standards of sterility, pyrogenicity, purity and general safety to the human patient systemically. Physical examination, tumor measurements, and laboratory tests should, of course, be performed before treatment and at intervals up to one to few months after the treatment and one skilled  
25 in the art would know how to conduct such routine procedures. Clinical responses may be defined by any acceptable measure. For example, a complete response may be defined by the disappearance of all measurable tumors within a given period after treatment.

### 30 EXAMPLES

The following examples further illustrate the present invention. The examples below are carried out using standard techniques, that are well known and routine to

those of skill in the art, except where otherwise described in detail. The examples are offered by way of illustration and not by way of limitation.

**EXAMPLE 1 Induction of Telomere shortening, G2 arrest and apoptosis in telomerase negative ALT cells after AZT or ganciclovir treatment**

To detect L1 specific RNA in two cell lines (U-2 OS and Saos-2 osteosarcomas), reported to maintain telomeres by ALT mechanism<sup>4</sup>, total mRNA was analyzed by dot blotting with an L1 retrotransposon specific probe. The reported telomerase-positive cell lines (HEC-1 and HeLa) were used for comparison<sup>4,21</sup> (Figure 1). Both ALT cell lines (U-2 OS and Saos-2 osteosarcomas) were positive in this test. HEC-1 cells were completely negative, with only traces of L1 transcripts in HeLa cells, as previously reported<sup>20</sup>.

The ALT cell lines were treated with therapeutic concentrations of AZT, to determine if slippage telomeric DNA synthesis could be inhibited by AZT-TP, and thereby induce telomere shortening. Telomere length in AZT treated and untreated cell lines was measured by flow cytometry with a telomere- specific peptide nucleic acid (PNA) probe<sup>22,23</sup>. To determine cell cycle distribution, cells were stained with propidium iodide (PI)<sup>22</sup>. After 14 days of AZT treatment, both ALT cell lines demonstrated telomere shortening, massive apoptosis and G2 arrest (Figure 2). To confirm the specificity of AZT-induced telomere shortening for ALT cells, a HeLa cell line, known to be positive for telomerase, was treated with AZT under the same conditions. AZT at the chosen concentration had no effect on telomere length or cell cycle distribution in the HeLa cells (not shown).

To demonstrate telomere shortening and changes in DNA synthesis rate, dynamic, U-2 OS cells were treated with AZT for different amounts of time, and analyzed by flow cytometry simultaneously. Rate of DNA synthesis was determined by incorporation of 5-bromodeoxyuridine (BdU)<sup>24</sup>. Results (Figure 3) show progressive telomere shortening and decrease in DNA synthesis. It is important to note that changes in cell cycle distribution, DNA synthesis and telomere length were rapid and could be detected after only 10 days of AZT treatment.

At the same time, PI staining demonstrated a higher DNA content in AZT treated cells at later stages of treatment, compared to untreated cells. A rational



explanation of this fact is a short telomere induced chromosome end-to-end joining<sup>12,26</sup>. Induction of apoptosis in AZT treated ALT cells seems to be p53 independent since U-2 OS and Saos-2 represent both p53+/+ and p53-/- cancer cell lines<sup>27</sup>.

5 Separately, U-2 OS cells were also treated with therapeutic concentrations of a guanine analog, ganciclovir (GCV), to demonstrate that the slippage telomeric DNA synthesis can be inhibited by GCV-TP and telomere shortening can be induced. Telomere length in untreated (Figure 4a) and GCV treated cells (Figure 4b) was measured by flow cytometry with a telomere-specific PNA probe as described above.

10 To determine cell cycle distribution, cells were stained with propidium iodide (PI). After 14 days of treatment with GCV at a concentration of 0.3 µg/ml, the U-2 OS cells demonstrated telomere shortening, massive apoptosis (programmed cell death) and G2 arrest.

It should be noted that the nucleoside analogs such as AZT and GCV are  
15 converted to their triphosphate forms once inside the host cell. For example, as is well known in the art, GCV is first phosphorylated to GCV-monophosphate (GCV-MP). GCV-MP is then further phosphorylated to GCV-biphosphate (GCV-BP) and GCV-triphosphate (GCV-TP) by endogenous kinases. GCV-TP lacks the 3' OH on the deoxyribose as well as the bond between the 2' and 3' carbons that are necessary  
20 for DNA chain elongation. Therefore, GCV-TP integration into the genome in the U-2 OS cell or other host cell inhibits DNA polymerase and causes DNA chain termination, which leads to apoptosis of the cell.

Tumors with suppressed elongation of telomeres have been reported to lose their tumorigenic potential<sup>12,26</sup>, and AZT is already in clinical use for treating AIDS.  
25 The present disclosure provides that AZT can be used for the treatment of up to 30% of cancer cases. Some other nucleoside reverse transcriptase inhibitors (e.g. 2',3'-dideoxyinosine (ddI) or 2',3'-dideoxy-3'-deoxythymidine(d4T)) that are already in clinical practice could also be used.

30 **EXAMPLE 2: Induction of Telomere shortening, G2 arrest and apoptosis in telomerase negative ALT cells after antisense inhibition of L1 reverse transcriptase**

To confirm that ALT is conducted by L1 reverse transcriptase only, U-2 OS cells were transfected expressing constructs containing part of human L1 ORF2 in sense and antisense orientation. The L1 specific reverse transcriptase targeted antisense construct was created as follows: PCR was performed using RT-F (5'-  
5 ATG ACA GGA TCA ACT TCA CAC -3') (SEQ ID NO:8), RT-R (5'- TCC TGC  
TTT CTC TTG TAG GCA -3') (SEQ ID NO:6) primers and pBS-L1<sub>RP</sub>-EGFP  
plasmid as a template. 929 bp PCR product was cloned in pTargetT vector (Promega).

Recombinant constructs containing insert in sense and antisense orientation were purified with Plasmid Midi Kit (Qiagen), digested with Xmn I (Promega) and  
10 transfected into U-2 OS cells using "Lipofectamine" (Gibco) according to the  
manufacturers instructions. After 40 days of selection on media containing 0.5 mg/ml  
of G418 (Gibco), cells were harvested, stained with PNA and PI, and analyzed by  
flow cytometry<sup>22</sup>. A schematic representation of L1 reverse transcriptase antisense  
targeting is shown in Figure 5.

15 Data presented in Figure 6 show that cells carrying antisense construct  
demonstrated massive apoptosis, G2 arrest, and telomere shortening as expected. In  
contrast, cells expressing sense construct showed no difference in telomere length or  
cell cycle.

The following materials and procedures were used in the above working  
20 examples:

**Cell lines:** All cell lines used in this study were obtained from American  
Type Culture Collection (Rockville, MD). The cells origins included osteosarcoma  
(Saos-2 and U-2 OS), liver (HEC-1) and uterine cervix (HeLa). Cells were cultured  
following ATCC recommendation. For treatment of the cells with AZT, the media  
25 was supplemented with 0.2  $\mu$ M of 3'-azido-2',3'-dideoxythymidine (Sigma)<sup>28</sup>.

**Dot blotting:** Total cellular RNA was isolated using "RNA-STA 60" solution  
(Tel-Test, Inc.). The reaction was performed using 30  $\mu$ g of total RNA and "HRP  
North2South" (Pierce) labeled pBS-L1<sub>RP</sub>-EGFP plasmid<sup>29</sup> as a specific probe,  
according to the manufacturers protocol.

30 **Bromodeoxyuridine incorporation:** Cell staining, for BrdU incorporation,  
was performed using cells which were incubated with 10 mM BrdU (Sigma) for 2.5 h,  
stained with BU-33 anti-BrdU monoclonal antibodies (Sigma) and FITC labeled

Alexa 488 goat anti-mouse IgG (H+L) (Fab') fragments (Molecular Probes), contrastained with 50 µg/ml PI (Sigma) and analyzed by flow cytometry as described<sup>24</sup>.

**Telomere length measurement by flow cytometry:** Cell were stained with  
 5 telomere specific FITC conjugated (C<sub>3</sub>TA<sub>2</sub>)<sub>3</sub> PNA (Applied Biosystems) probe and contrastained with 0.06 µg/ml PI as described<sup>21</sup>.

**Inhibition of L1 reverse transcriptase using antisense strategy:** To create L1 specific reverse transcriptase targeted antisense construct PCR was performed using RT-F (5'-ATG ACA GGA TCA ACT TCA CAC-3') (SEQ ID NO:8), RT-R  
 10 (5'-TCC TGC TTT CTC TTG TAG GCA-3') (SEQ ID NO:6) primers and pBS-L1<sub>RP</sub>-EGFP plasmid as a template. 929 bp PCR product was cloned in pTargetT vector (Promega). Recombinant constructs containing insert in sense and antisense orientation were purified with Plasmid Midi Kit (Qiagen), digested with Xmn I (Promega) and transfected into U-2 OS cells using "Lipofectamine" (Gibco)  
 15 according to the manufacturers instructions. After 40 days of selection on media containing 0.5 mg/ml of G418 (Gibco), cells were harvested, stained with PNA and PI, and analyzed by flow cytometry<sup>22</sup>.

The references numbered 1-29 below are cited in the above description (with the corresponding superscript numbers) and as such one skilled in the art would match  
 20 the references to the appropriate superscript numbers in the text above.

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All publications, patents and patent applications mentioned in the specification are indicative of the level of those skilled in the art to which this invention pertains. All publications, patents and patent applications are herein incorporated by reference

5 to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference. Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.